POLYPEPTIDE MOLECULES OF THE PRE-ERYTHROCYTIC STAGE OF MALARIA

The parasites responsible for malaria in man display different morphologies in the human host and express different antigens depending on their location in the body. The morphological and antigenic differences of these parasites during their life cycles in man enable different stages of development in the liver and in the blood to be defined: the sporozoite, the infectious form injected by the vector mosquito, transforms rapidly into a schizont in the host's hepatocytes and thereafter infects the erythrocytes. The intrahepatic localization of P.falciparum manifests itself in the expression of a group of antigens specific to this stage of development and which are highly immunogenic under the natural conditions of exposure to the disease. This clinically silent phase is at present the only one against which a very strong, sterilizing immunity can be induced experimentally in man, by injecting irradiated sporozoites capable of entering the hepatocyte and of developing therein but without being able to lead on to the blood stage of the disease. Accordingly, the inventors have concentrated the bulk of their efforts on these two pre-erythrocytic stages. However, these stages are also the most intricate ones to study, and hence the least understood, since it is difficult or even impossible to obtain biological material, the only in vitro study model affords a very low yield and the best animal

model remains the chimpanzee, the use of which is limited and expensive.

In order to gain access to the antigens of the pre-erythrocytic stages, the inventors used sera of individuals who had resided for 25 years in a region where the disease is endemic but who were on permanent prophylaxis with chloroquine. These individuals were regularly subjected to infected mosquito bites but did not develop any complete blood infection. Their serum hence contained antibodies directed essentially against the pre-erythrocytic stages, which was verified by immunofluorescence (IF) and western blotting on the 3 stages of the parasite.

The use of these sera for screening a library of genomic DNA of the parasitic clone of P.falciparum, the library being constructed in expression vectors in a phage lambda gt11 (V. Rosario, Science 212, 1981, pp. 1037-1038; and Thaithong et al., Transactions of Royal Society of Tropical Medicine and Hygiene, 1984, 78:242-245), led to the demonstration of polypeptides of the pre-erythrocytic stage, in particular the SALSA (sporozoite liver stage antigen) polypeptides described in EP A-0,407,230 and LSA-1 (liver stage antigen) described in WO 92/13884. The present invention relates to new polypeptide molecules specific to the pre-erythrocytic stage, and to their use as active principle of antimalarial vaccine or in methods of diagnosis of the disease.

The invention is the outcome of the demonstration by the inventors of the special properties of a particular antigen referred to as LSA-3 and of its fragments, which are seen to be candidates with a strong potential for producing an antimalarial vaccine, for the following reasons:

a) when a fraction of LSA-3 was used in combination with another antigen of the same stage of development of the parasite, such as LSA-1, to immunize chimpanzees, the animal responding to both molecules or only to LSA-3 displays the feature of not having

parasites in the blood, of having a substantial decrease of the parasites in the liver and of manifesting a substantial recruitment of mononuclear cells indicating a response in terms of cellular immunity;

b) in regions where the disease is endemic, a very clear correlation is observed between the protection of individuals against natural infection by sporozoites and their responses in terms of antibodies against LSA-3;

c) in eight human volunteers immunized by injection of irradiated sporozoites, antibodies directed against LSA-3 are found in each of the four individuals resisting sporozoite infection and in none of the other four volunteers who developed a blood infection;

d) antibodies obtained against the peptide DG729 in WO 92/13884, already described, give a cross-reaction with the sporozoite and liver stages of the murine parasite P.yoelii, which permits a significant exploitation of the mouse model. In vitro, the human antibodies immunopurified on DG729 are capable, even at very low concentrations, of blocking the entry of P.yoelii sporozoites into mouse hepatocytes. In vivo, mice immunized with DG729 are fully or partially protected against infection by P.yoelii sporozoites;

e) lastly, some epitopes, in particular in the non-repeat portions of the molecule, stimulate the secretion of interferon-γ by monocytes, this mediator enabling the intrahepatic development of the parasite to be inhibited (S. Mellouk et al., The Jour. Of Immun. 139, 4192-4195, 1987);

f) the sequence of the region of LSA-3 corresponding to a (lipo)peptide NR2 was analysed in 27 samples: 4 laboratory strains (NF54, K1, Palo Alto, T9/96), 3 Madagascan isolates, 3 Burmese isolates, 5 Brazilian isolates, 7 isolates from the Ivory Coast and 5 Thai isolates. No mutation was observed on the 300 base pairs analysed, that is to say 100% conservation in this immunologically important region containing one or more B, Th and CTL epitopes;

g) information about the structure of the antigen, and in particular of a peptide RE, and more especially about the central repeat region from which the peptide RE was designed and which contains one or more major B epitopes, was obtained from the hydrophobic cluster plot of the sequence available in the clone T9/96 (630 amino acids) (Gaboriot et al., (1987): Hydrophobic cluster analysis: an efficient new way to compare and analyse amino acid sequences, FEBS Letters, 224: 149-155); this method predicts a very strong propensity for α-helical organization. The repeat region displays remarkable regularity in the spacing of the valine and isoleucine residues, alternating with acid or proline residues. The arrangement of the hydrophobic groups at the surface of this helix is reminiscent of a hydrophobic border gradually shifting from one face of the helix to the other according to a constant general orientation along the molecule, and probably related to a coiled-coil structure or packaging as seen in Figure 4b which depicts the HCP (hydrophobic cluster plot) of the peptide sequence of the clone DG729;

h) after demonstrating that there was a very wide range of immune responses to the LSA-3 antigen, we analysed the capacity of the responder cells to localize around the parasites in the liver. In mice immunized with the recombinant antigens, intraportal injection of each of the peptides absorbed on 10 μm polystyrene beads enables an afflux of lymphocytes around the antigen (mimicking the parasite) to be visualized after 48 hours, followed on the 5th day by a substantial recruitment of cells belonging to the macrophage line.

All these properties, some of which will be demonstrated in detail in the experiments described later, show that the LSA-3 antigen displays both good antigenicity and good immunogenicity.

The inventors were able to confirm and define the specificity of the stages of expression of the molecule; in the sporozoites, this expression was confirmed by the surface immunofluorescence of several

strains and isolates. In western blot (or immunoblot) analysis, the LSA-3 molecule appears as a protein of molecular weight 200,000 daltons. While the messenger RNAs of sporozoites could not be obtained in sufficient amounts for a northern blot analysis, reverse PCR experiments confirmed the expression of LSA-3 at this stage. In infected hepatocytes, LSA-3 is observed in the parasitophorous vacuole of the parasite by immuno-fluorescence using antibodies against the repeat and non-repeat regions of the protein, as well as by electron microscopy.

A fragment of LSA-3 designated 729S, as well as three peptides designated NRI and NRII included in the non-repeat portion and 729R included in the repeat portion, have been described in Application WO 92/13884. Nevertheless, this document does not mention the special properties mentioned above, or other fragments of LSA-3 which could be either longer or shorter, included or combined with these fragments, which might display especially advantageous properties for use in vaccines.

The subject of the invention is polypeptide molecules containing at least ten consecutive amino acids of the amino acid sequence shown in Figure 2 and designated SEQ ID No. 2, and representing LSA-3, the following polypeptides being excluded:

- RDELFNELLNSVDVNGEVKENILEESQVNDDIFNSLVKSVQQEQQHNVEE
- VEESVEENDEESVEENVEENVENNDDGSVASSVEESIASSVDESIDSSIE-ENVAPTVEEIVAPTVEEIVAPSVVEKCAPSVEESVAPSVEESVAEMLKER (729S)
- RDELFNELLNSVDVNGEVKENILEESQVNDDIFNSLVKSVQQEQQHN
- DELFNELLNSVDVNGEVKENILEESQ, (NRI)
- LEESQVNDDIFSNSLVKSVQQEQQHNV, (NRII)
- VESVAPSVEESVAPSVEESVAENVEESV. (729RE)

Other molecules according to the invention contain at least 20 consecutive amino acids or at least 50.

This set of polypeptides and the LSA-3 molecule are, throughout hereinafter, "polypeptides of the invention".

The experimental results and the comparisons of non-repeat sequences between different P.falciparum isolates indicate the existence of at least 70% homology between equivalent antigens of the liver stage of the parasite. Thus any peptide molecule displaying at least 70% homology with any one of the molecules defined above forms part of the invention, as do those displaying at least 70% homology with the following sequence:

Leu Leu Ser Asn Ile Glu Glu Pro Lys Glu Asn Ile Ile Asp Asn Leu Leu Asn Asn Ile (CT1)

lying between amino acids 140 and 159 of K1 or 23 and 42 of T9/96.

Likewise forming part of the invention are the polypeptide molecules displaying at least 70% homology with the sequence depicted in Figure 3, which depicts a portion of LSA-3 in T9/96: the DNA of this P.falciparum isolate was digested with restriction enzymes, then cloned into lambda gt11 and thus enabled the gene library of this isolate, already described above, to be constituted.

Conjugates consisting of a polypeptide originating from LSA-3 linked covalently via a lysine bridge to saturated or unsaturated lipid residues also form part of the invention, more especially when the lipid residue is a palmitoyl or a palmityl or an oleyl. C_{16} or C_{18} residues were thus coupled via a lysine bridge to the peptides NRI, NRII, 729RE and CT1 already depicted above. The method of synthesis used for these conjugates is described in Bourgault, Journal of Immunology, 149, 3416 (1992) and Rouaix, Vaccine, 12, 1209 (1994).

The invention also covers immunogenic compositions containing at least one polypeptide molecule or one conjugate described above, as well as the vaccines containing these immunogenic compositions. Other

immunogenic epitopes, in particular LSA-1, SALSA and STARP, have already been described in EP A-0407230 and in WO 92/13884. The vaccine compositions according to the invention can advantageously contain a mixture of immunogenic peptides originating from LSA-3 and of the peptides or antigens originating from LSA-1, SALSA or STARP; a more especially advantageous mixture could be the one consisting, on the one hand of NRI, NRII or whole LSA-3, these being coupled or otherwise to a lipid residue, and on the other hand the peptides SALSA-1, SALSA-2 or the SALSA antigen coupled or otherwise to a lipid residue.

All polypeptide molecules corresponding to the above definition and displaying at least 70% homology with the polypeptides LSA-3, CT1, NRI, NRII or 729RE may be combined in homologous or heterologous fashion with other peptide sequences or sequences originating from another antigen of the different stages of P.falciparum.

70% Homology of sequences should be clearly understood to refer to a sequence homology with respect to any one of the isolates whose sequence is known or capable of being known, and not an overall homology between the collective isolates. In effect, the central repeat region of LSA-3 (block 2 of Figure 4) displays a variable number of repeat sequences responsible for a variability from one isolate to another, as seen, moreover, in the diagram of Figure 4, in which the difference in length between the repeat portions of block 2 of the isolates T9/96 and K1 is blatant although the tetrapeptides which constitute this repeat region (VEES, VEEN, VEEI, VAPS, VAPT and the like) are very well conserved. In contrast, the repeat sequences of block 1 are fully conserved between the two isolates. Thus, bearing in mind the intrinsic variability of this block 2 from one isolate to another, the definition 70% homology applies to the LSA-3 antigen of the different isolates excluding the repeat sequences of block 2.

The invention also covers the polyclonal or monoclonal antibodies which specifically recognize the polypeptide molecules of the invention.

These molecules of the invention may be used for carrying out diagnostic methods and producing kits enabling the existence of P.falciparum infection to be detected; this method can be either an assay of circulating specific antibodies, by carrying out standard serological methods by bringing one of the above antigens into contact with a biological fluid of the individual in question, or methods of assay of antigens using polyclonal or monoclonal antibodies obtained by standard methods for obtaining such antibodies with the corresponding antigens. In the diagnostic outfits or kits of the invention, the reagents enabling the antigen/antibody complexes produced to be detected, which can also carry a label or be capable of being recognized in their turn by a labelled reagent, are present. Depending on whether it is desired to carry out an antigen test or a serological test, the kit comprises either the antibodies or the antigens of the invention.

The invention also covers all the nucleotide sequences coding for a polypeptide of the invention, as well as any recombinant nucleic acid containing at least one nucleotide sequence of the invention, inserted into a nucleic acid which is heterologous with respect to the said nucleotide sequence.

The nucleic acid sequences coding for LSA-3 or its immunogenic fragments and corresponding to one of the following definitions form part of the invention:

(a) the linked succession of nucleotides as depicted in SEQ ID No. 1 of Figure 1, or

(b) the linked succession of nucleotides depicted in SEQ ID No. 2 of Figure 2,

(c) a linked succession displaying at least 70% homology with that of Figure 1 or of Figure 2, or

(d) a linked succession of nucleotides which are complementary to those presented in (a), (b) or (c).

The expression "coding for LSA-3" is understood to refer both to the gene depicted in SEQ ID No. 1 of Figure 1 and the cDNA depicted in SEQ ID No. 2 of Figure 2.

The invention relates more especially to a recombinant nucleic acid in which the nucleotide sequence of the invention is preceded by a promoter (in particular an inducible promoter), under the control of which the transcription of the said sequence is capable of being performed, and, where appropriate, followed by a sequence coding for transcription termination signals.

The invention also covers the coding sequence originating from the clone T9/96 depicted in Figure 3 by SEQ ID No. 3.

In this sequence, the fragment CT1 lies in [sic] nucleotide [sic] 67 and 126, the fragment 679 how [sic] at nucleotide 206 and the fragment 729RE lies between nucleotides 547 and 630.

Lastly, the invention covers any recombinant vector used especially for the cloning of a nucleotide sequence of the invention, and/or for the expression of the polypeptide encoded by this sequence, and characterized in that it contains a recombinant nucleic acid as defined above in one of its sites which is not essential for its replication.

As an example of an abovementioned vector, plasmids, cosmids, phages or viruses may be mentioned.

As such, the invention relates more especially to the plasmid pK 1.2. deposited at the CNCM under the No. I-1573.

The subject of the invention is also a method for preparing a polypeptide of the invention, by transformation of a cell host using a recombinant vector of the abovementioned type, followed by the culturing of the cell host thus transformed and the recovery of the polypeptide in the culture medium.

Thus, the invention relates to any cell host transformed by a recombinant vector as defined above,

and comprising the regulatory elements permitting the expression of the nucleotide sequence coding for a polypeptide according to the invention.

The invention likewise covers DNA (or RNA) primers which can be used in the context of the synthesis of nucleotide and/or polypeptide sequences of the invention, by the PCR (polymerase chain reaction) technique or any other method known at the present time for amplifying nucleic acids, such as LCR, CPR, ERA, SPA, NASBA, and the like.

The invention relates to any DNA or RNA primer, characterized in that it consists of approximately 10 to 25 nucleotides which are identical or complementary to the first 10 to 25 nucleotides of the nucleotide sequence coding for a peptide sequence according to the invention, or identical to the last 10 to 25 nucleotides of the said sequence.

Thus, the present invention also covers a method for preparing a polypeptide of the invention comprising the following steps:

- where appropriate, the prior amplification by standard techniques of the amount of nucleotide sequences coding for the said polypeptide using two suitably chosen DNA primers,
- the culturing, in a suitable culture medium, of a cell host previously transformed by a vector containing a nucleic acid according to the invention comprising the nucleotide sequence coding for the said polypeptide, and
- the recovery from the abovementioned culture medium of the polypeptide produced by the said transformed cell host.

By way of example of DNA or RNA primers according to the invention, the following pairs of sequences may be mentioned:

S1: GTGATGAACTTTTTAATGAATTATTAAA (SEQ ID No. 4)

S2: TGTTGTTCTTGTTGAACACTTTTTACTAA (SEQ ID No. 5)

whose respective positions on the LSA-3/K1 gene depicts [sic] in Figure 1 are from 695 to 722 and from 829 to 799 (reading in the reverse direction), or the pair:

6.1: GGTATCGAAACTGAGGAAATAAAGG (SEQ ID No. 6)

6.2: CATAGCAGGAACATCAACATCCAC (SEQ ID No. 7)

whose respective positions are 2668 to 2692 for 6.1 and 3456 to 3433 for 6.2 (reading in the reverse direction).

The information regarding the sequences ID No. 4, ID No. 5, ID No. 6 and ID No. 7 are detailed at the end of the description.

The peptides of the invention may also be prepared by the standard techniques of peptide synthesis. This synthesis may be carried out in homogeneous solution or in the solid phase. For example, use may be made of the technique of synthesis in homogeneous solution described by Houben-Weyl in the work entitled "Methoden der Organischen Chemie" (Methods in Organic Chemistry) edited by E. Wunsch, vol. 15-I and II. Thieme, Stuttgart 1974, or that described by R.D. Merrifield in the paper entitled "Solid phase peptide synthesis" (J. Am. Chem. Soc., 45, 2149-2154).

The invention also covers the water-soluble oligomers of the abovementioned monomeric peptides.

Oligomerization can cause an enhancement of the immunogenicity of the monomeric peptides according to the invention. While such numerical information cannot be regarded as limiting, it may nevertheless be mentioned that these oligomers can, for example, contain from 2 to 10 monomer units.

To carry out the oligomerization, use may be made of any polymerization technique commonly used in the peptide field, this polymerization being conducted until an oligomer or polymer containing the requisite number of monomer motifs for acquiring the desired immunogenicity is obtained.

One method of oligomerization or polymerization of the monomer consists in reacting the latter with a crosslinking agent such as glutaraldehyde.

Use may also be made of other oligomerization or coupling methods, for example the one employing successive couplings of monomer units via their carboxy- and amino-terminal functions in the presence of homo- or heterobifunctional coupling agents.

The invention also relates to the conjugates obtained by covalent coupling of the peptides according to the invention (or of the abovementioned oligomers) to physiologically acceptable and non-toxic (natural or synthetic) carrier molecules that enable, in particular, the immunogenicity to be argued [sic], via complementary reactive groups carried, respectively, by the carrier molecule and the peptide. By way of example of macromolecular carrier molecules or supports which participate in the constitution of the conjugates according to the invention, there may be mentioned natural proteins such as tetanus toxoid, ovalbumin, serum albumins, haemocyanins, tuberculin PPD (PPD: purified protein derivative), and the like.

By way of synthetic macromolecular supports, my [sic] may be mentioned, for example, polylysines or poly(DL-alanine)-poly(L-lysine)s.

By way of hydrocarbon or lipid supports, there may be mentioned saturated or unsaturated fatty acids, and preferably C_{16} or C_{18} acids of the oleyl [sic] or palmitoleyl [sic] type.

Lastly and without implied limitation, the antigens or peptides according to the invention may be coupled to traditional supports or adsorbed on such supports, in particular latex or polystyrene microspheres or beads, or incorporated in Ty1 particles.

To synthesize the conjugates according to the invention, use may be made of methods which are known per se, such as the one described by Frantz and Robertson in Infect. and Immunity, 33, 193-198 (1981), or the one described in Applied and Environmental

Microbiology (October 1981), vol. 42, No. 4, 611-614 by P.E. Kauffman, using the peptide and the appropriate carrier molecule.

The nucleic acids of the invention may be prepared either by a chemical method or by other methods.

A suitable method of preparing the nucleic acids of the invention containing not more than 200 nucleotides (or 200 bp in the case of double-stranded nucleic acids) comprises the following steps:

- DNA synthesis using the automated β-cyanoethyl-phosphoramidite method described in Bioorganic Chemistry 4; 274-325 (1986),
- cloning of the nucleic acids thereby obtained into a suitable vector and recovery of the nucleic acid by hybridization with a suitable probe.

A chemical method of preparation of nucleic acids of length greater than 200 nucleotides has already been described in WO 92/13884.

The invention also relates to diagnostic kits which contain one or more amplification primers specific for the LSA-3 gene and which enable the presence of the gene or of the mRNA to be detected in an individual likely to be infected by P.falciparum.

The invention also covers pharmaceutical or vaccine compositions in which at least one of the products according to the invention is present in combination with solid or liquid, pharmaceutically acceptable excipients suitable for the construction of oral, ocular or nasal dosage forms, or excipients suitable for the construction of dosage forms for rectal administration, or alternatively with gelatinous excipients for vaginal administration. It also relates to isotonic liquid compositions containing at least one of the conjugates according to the invention, suitable for administration to the mucosae, in particular the ocular or nasal or pulmonary mucosae.

Advantageously, the vaccine compositions according to the invention contain, in addition, a

vehicle such as polyvinylpyrrolidone which facilitates the administration of the vaccine. In place of polyvinylpyrrolidone, it is possible to use any other type of adjuvant, in the traditional sense which was formerly given to this expression, that is to say a substance which enables a medicinal product to be absorbed more readily or which facilitates its action in the body. By way of examples of other adjuvants of this latter type, there may also be mentioned carboxymethylcellulose, aluminium hydroxides and phosphates, saponin or all other adjuvants of this type which are well known to a person skilled in the art. Lastly, they contain, if necessary, an immunological adjuvant, in particular of the muramyl peptide type.

The invention also relates to pharmaceutical compositions containing as active substance at least one of the polyclonal or monoclonal antibodies defined above, in combination with a pharmaceutically acceptable vehicle.

Lastly, the invention covers a method of immunization of an individual likely to be infected by P.falciparum, by injection of a peptide molecule or an oligomer as described above, alone or in combination with other types of molecules capable of protecting the said individual against subsequent infection; the polypeptide or antigenic molecule or the natural or recombinant lipopeptides are either used alone or adsorbed or coupled to latex or polystyrene microspheres or beads.

Additional features of the invention will also become apparent in the examples illustrated with the figures which follow, and show the special features of the molecules of the invention relative to other antigens of the pre-erythrocytic stage of the parasite.

Figure 1 depicts the genomic DNA sequence ID No. 1 of 6152 base pairs of the LSA-3 gene; it originates from the clone K1.2, which itself originates from a Thai isolate.

Figure 2 depicts the cDNA sequence ID No. 2 and the polypeptide sequence of the LSA-3 antigen. The DNA sequence represents 5361 base pairs.

Figure 3 depicts the sequence ID No. 3 of the portion sequenced in the parasite clone T9/96 (1890 base pairs), the upper line being the nucleotide sequence and the lower line the peptide sequence. In this clone, the CT1 sequence lies between nucleotides 67 and 126, the actual fragment DG679 beginning at nucleotide 207. The fragment 729RE lies between nucleotides 547 and 629.

Figure 4a depicts diagrammatically the relative positions of the repeat and non-repeat sequences, the introns and the exons in strains K1 and T9/96, the clones 679 and 729 originating from the latter.

Figure 4b depicts the HCP (hydrophobic cluster plot) of the peptide sequence of the clone DG729.

Figure 5 depicts the amounts of immunoglobulins produced in the serum of chimpanzee Nuria before and after immunization with different LSA-3 peptides.

Figure 6 shows the specific antibody titre of different species of mice immunized either with a peptide or with a corresponding lipopeptide.

Figure 7 shows the inhibition of the sporozoite invasion of liver cells by hyperimmune sera obtained after immunization with different peptides [lacuna] immunopurified against whole LSA-3.

Figure 8 depicts the comparison of an antigen originating from LSA-3 with two other antigens with respect to type T immunity.

Figure 9 depicts the induction of interferon-γ in the chimpanzees Gerda and Dirk with the peptides originating from the LSA-3 molecule.

Figure 10 depicts the results of lymphoproliferation of the PBMC of an individual protected by an injection of irradiated sporozoites against peptides originating from the LSA-1 and LSA-3 antigens.

Example 1: Cloning and sequencing of the LSA-3 gene

1) Sequencing

Initial screening of the gene library originating from the parasite clone T9/96 with the serum of a missionary treated continuously by prophylaxis enabled us to isolate 120 clones corresponding to molecules expressed at the sporozoite and/or liver stage of the P.falciparum cycle. The clone 729S was used as probe to screen a genomic library of the Thai strain K1 already mentioned above, which contains large EcoR I fragments cloned into phage lambda gt10. A 6.85-kilobase insert containing the whole gene was purified from this gene library and recloned into a pUC18 plasmid for sequencing and characterization. In P.falciparum, the genome of which is very rich in bases A:T(80%), this approach is often rendered difficult by the rarity of restriction sites which can be used, and by the instability or even the impossibility of cloning certain fragments when they are inserted into plasmid vectors.

The structure of the gene is depicted in Figure 4 and displays the following features:

a) a mini-exon 1 coding at its 3' end for a hydrophobic signal peptide;

b) a short intron (168 base pairs) included between consensus splicing donor and acceptor sites;

c) a second exon of five kilobases which codes for an organized region of 1.8 kilobases, and composed of an arrangement of 7 blocks of 4 amino acids and a 3' hydrophobic region which might correspond to an inking [sic] of the glycosylphosphatidylinositol (GPI) type.

A detailed investigation of the polymorphism of LSA-3 was carried out by sequencing the clone 679, which contains the bulk of the repeat sequences of the LSA-3 gene and a 1-kilobase portion of the 3' non-repeat fraction, the sequence of this fragment being depicted in Figure 3 between nucleotides 207 and 1890.

The strain K1 repeats are the following:

Block 1: (aa223) VEEK VEES VEEN DEES VEEN VEEN VEEN DDGS VASS VEES IASS VDES IDSS IEEN (aa278)

Block 2: (aa279) VAPT VEEIVAPS VVESVAPS VEESVEEN VEESVAEN VEESVAEN VEESVAEN VEESVAEN
VEEIVAPT VEEIVAPT VEEIVAPS VVESVAPS VEESVEEN
VEESVAEN VEESVAEN VEESVAEN VEESVAEN VEESVAEN
VEEIVAPT VEEIVAPT VEEIVAPS VVESVAPS VEESVEEN
VEESVAEN VEESVAEN VEESVAEN VEESVAEN VEESVAEN
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VEEIVAPT VEEIVAPT VEEIVAPS VVESVAPS VEESVEEN
VEESVAEN VEESVAEN VEESVAEN VEESVAEN
VEEIVAPT VEEIVAPT VEEIVAPS VVESVAPS VEESVEEN
VEESVAEN VEESVAEN VEESVAEN
VEEIVAPT VEEIVAPT VEEIVAPS VVESVAPS VEESVEEN
VEESVAEN VEESVAEN VEESVAEN VEESVAEN
VEESVAPT VEEIVAPS VEESVAPS VEESVAEN (aa818)

Block 3:
(aa1537)DEDI EEDV EEDI EEDI EEDK VEDI DEDI DEDI GEDK DEVI (aa1576)

The repeats in the clone T9/96 as determined in Patent Application No. FR 91/01286 of 5th February 1991 are the following:

Block 1: VEEK VEES VEEN DEES VEEN VEEN VEEN DDGS VASS VEES IASS VDES IDSS IEEN

Block 2: VAPT VEEIVAPT VEEIVAPS VVESVAPS VEESVAPS VEESVAEN VEESVAEN
VEEIVAPS
VEESVAEN VEESVAEN VEESVAEN VEESVAEN VEESVAEN
VEEIVAPT VEESVAPT VEEIVAPT VEESVAPT VEEIVVPS VEESVAPS
VEESVAEN VEESVAEN VEESVAEN VEESVAEN VEESVAEN
VEEIVAPS VEEIVAPT
VEESVAEN

Exon 2 of the LSA-3 gene contains 2 repeat regions which can be split into 3 blocks as shown in Figure 4:

- Block 1, coding for a linked succession of 14 tetrapeptides. This block is 100% conserved with respect to amino acids and nucleic acids between T9/96 and K1. Only the tetrapeptides VEES and VEEN are to be found in block 2.

- Block 2 codes, in K1, for 127 tetrapeptides corresponding to the linked succession of different octapeptides which are themselves formed by combination of 2 of the 7 basic tetrapeptides or motifs (VEES, VVES, VEEN, VEEI, VAEN, VAPS, VAPT). The number of repeats and the arrangement of these octapeptides vary according to the motifs and appear to be specific to the clone K1.2. In effect, in the clone T9/96, block 2 (53 tetrapeptides) also corresponds to the linked succession of octapeptides formed from the same 7 basic tetrapeptides (and an 8th motif VVPS which does not exist in K1), but with a different number and arrangement of these repeats.

- Block 3 consists of the linked succession of 10 degenerate tetrapeptides different from those of blocks 1 and 2. This block was sequenced only in the strain K1. However, preliminary results obtained by PCR with the clone T9/96 and several other laboratory strains indicate that there is no size polymorphism in this region.

The non-repeat regions of exon 2 are especially well conserved. In effect, sequence comparison between T9/96 and K1 could be done on 315 bp at the 5' end of block 1 and on 763 bp at the 3' end of block 2. The homology is 99.4% with respect to nucleic acids and 98.6% with respect to amino acids.

Comparison of the sequences of the clone 679 originating from P.falciparum clone T9/96, and of the corresponding sequence of LSA-3 originating from the isolate K1, shows that the gene is well conserved, the most significant differences being observed in the

repeat region where the blocks of 4 amino acids are well conserved but vary in their number and organization.

In contrast, the non-repeat 5' and 3' portions apear to be especially well conserved, showing up to 100% homology in the 5' region where B and T epitopes have already been identified.

DNA amplifications, in particular by PCR of different P.falciparum strains with 8 primer pairs distributed over the whole of the LSA-3 gene, showed that, except with the ones surrounding the repeat regions, the whole of the genome gives PCR products of similar size, suggesting that the LSA-3 antigen is well conserved.

Various LSA-3 probes, chosen in the repeat and non-repeat regions, were hybridized at low stringency with the DNAs of different species of Plasmodium, and did not enable any gene homologous to LSA-3 to be identified except in the chimpanzee parasite P.reichenowi, confirming the close kinship of this species with P.falciparum.

Surprisingly, the antigen analogous to LSA-3 found in P.yoelii, which gives clear immunological cross-reactions at the surface of the sporozoite with antibodies against the fragment 729S, does not appear to be conserved at the level of the nucleotide sequence. Lastly, comparison of the LSA-3 sequences with the data bases did not reveal any homology with known molecules, except for the repeat region, some of the motifs of which display a strong analogy with the repeats of a Staphylococcus xylosis gene, but also with two P.falciparum antigens, RESA and Pf11.1, which are both expressed during the blood stage of the parasite. This homology is essentially due to the large amount of "Glu-Glu" sequences in these antigens and in the repeats of LSA-3.

2) Cloning

The insert DG729 and other regions of exon 2 of the strain K1 were cloned into a prokaryotic expression

vector pGEX, a vector marketed by the company InVitrogen Corp (San Diego USA). This vector produces a fusion protein with the Schistosoma mansoni glutathione S-transferase (GST), and enables the recombinant proteins to be purified readily by affinity for glutathione-agarose beads. The expression peptides from these vectors are designated:

- for the whole LSA-3 protein: REC protein,
- or for the fragment 729S:729PGEX.

Attempts at cloning other fragments, in particular the fragment 1-5 3NSREP, 3NFREP, 5NR and 5SNREP, caused difficulties related either to the cloning or to the production and purification of the proteins in sufficient amounts for immunization experiments.

Only the fragments 729, NN and 3PC enabled corresponding recombinant polypeptides to be produced and purified in sufficient amounts for analysis of the antigenicity of the molecule.

Example 2: Protection of immunized chimpanzees against challenge injections [sic] at low or high dose

2.1. A chimpanzee Dirk previously immunized with a fraction of LSA-3 in combination with another antigen of the same stage of development of the parasite, and displaying the effects described above in point a), was reimmunized a few years later with peptides and recombinant proteins corresponding to the same combination of antigens. Once again, this chimpanzee proves to be protected against a challenge infection at low dose (2×10^4 sporozoites) and then a challenge infection at high dose (5×10^6 sporozoites). As during the first challenge, a substantial reduction is observed in the number of schizonts detected in the liver after the challenge at high dose, as well as a lymphocytic-monocytic infiltrate around the few schizonts that are detectable (testifying to a local defence).

2.2 Partial protection of the chimpanzee Gerda: another chimpanzee was immunized only with the LSA-3

antigen (animal described in Examples 7 and 8 below), namely the lipopeptide NR2 and then recombinant proteins (GST-729, GST-NN, GST-3PC) which, the three of them collectively, cover 95% of the LSA-3 molecule and which are adsorbed on latex microspheres. This animal proves to be partially protected against a challenge infection at high dose (8×10^6 sporozoites), since it displays a very low blood parasitaemia and a 90% reduction in the number of liver schizonts relative to the control following the challenge infection.

2.3. Partial protection of the chimpanzee Nuria: a chimpanzee immunized with a fraction of the LSA-3 antigen alone, namely a combination of peptides, of lipopeptides and then of recombinant proteins corresponding to 95% of the LSA-3 molecule and emulsified in Montanide ISA-51 (SEPPIC, 75 Quai d'Orsay, France), proves to be partially protected against a challenge infection at moderate dose (1×10^5 sporozoites). In effect, this animal displays a significant delay in the appearance of the parasites in the blood relative to 4 controls (chimpanzees immunized with the pre-erythrocytic antigens LSA-1, SALSA or STARP, and 1 unimmunized control animal), a lower maximum blood parasitaemia and a faster fall in parasitaemia (24 hours instead of 3 days), which results reflect a large reduction in the number of liver forms induced in this animal by the challenge infection and in agreement with the results obtained in Gerda. In this case, examination of the liver forms was not carried out.

2.4. B and T immunogenicity in the chimpanzees Demi, Karlien and Iris: three chimpanzees immunized with the peptides LSA-3-NR1 and -RE and the lipopeptides -NR2 and -CT1, as well as with peptides corresponding, for each animal, to another pre-erythrocytic antigen (LSA-1, SALSA or STARP), display, all 3 of them:

- high humoral responses against the B epitopes present on the peptides NR1, NR2 and RE. The antibodies

recognize not only the peptides and the recombinants but are also strongly positive on the native molecules of the parasite, which is assessed by immunofluorescence on the sporozoites and the liver stages of Plasmodium falciparum (but negative with respect to the erythrocytic stages);

- high and specific lymphoproliferative responses against the 4 LSA-3 peptides, as well as the native T epitopes present at the surface of the sporozoites of Plasmodium falciparum and of Plasmodium yoelii, in which LSA-3 possesses a homologue (not yet characterized).

The B and T responses with respect to the native antigens are an important point since:

a) they prove that the synthetic molecules are indeed representative;

b) they signify that, at the time of infection, there are good prospects for obtaining an anamnestic secondary response; this is, in fact, what was observed in the chimpanzee Nuria at the time of the challenge. The importance of this observation is enhanced by the fact that the same secondary response was not obtained in respect of the other antigens such as LSA-1 and STARP.

2.5. Immunogenicity in Aotus: an owl monkey (Aotus trivirgatus) immunized with the 2 peptides LSA-3-NR1 and -RE and the 2 lipopeptides -NR2 and -CT1, and then restimulated with the recombinant proteins corresponding to 95% of the LSA-3 molecule and adsorbed on microspheres as described above, displays high and specific lymphoproliferative responses against the T epitopes present on these same peptides.

As regards the in vivo response of the different chimpanzees preimmunized in this way, the results underline the excellent immunogenicity (B and T) of LSA-3 in peptide, lipopeptide and recombinant form, and in all the animal models tested to date, namely 6/6 (outbred) chimpanzees and 1/1 Aotus, and in all the immunized mice (>20). It may be noted that the

results of the lipopeptide formulations (which can be used in man) were obtained by subcutaneous injection in the absence of any adjuvant.

Example 3: Identification of CTL epitope

The method used to identify CTLs is the one described by Fidock et al., (1994), J. Immunol. 153: 190, or by Bottius et al., (1996), J. Immunol. 156: 2874-2884.

CTL (cytotoxic T lymphocyte) epitopes were identified in the peptides NR2, RE and CT1 by means of cytotoxicity tests performed on the PBMCs of the chimpanzees Dirk, Gerda, Nuria, Demi, Karlien and Iris described above.

In man, 8 additional CTL epitopes, 7 of them located in the 3' non-repeat region, could be demonstrated on the PBMCs of individuals belonging to 3 different haplotypes (MHC class I-A2, -B8 and -B53) and living in a region where the disease is endemic (Gambia) (unpublished results). Furthermore, sequencing of the 2 B53-restricted CTL epitopes demonstrated a complete conservation of their nucleotide and peptide sequences in several strains from Kenya and from Gambia.

In total, we identified 11 CTL epitopes in the LSA-3 molecule, which is considerable. Moreover, 5/6 chimpanzees developed CTL responses against the peptide NR2 after immunization with the lipopeptide NR2 without adjuvant, which is a remarkable result for non-consanguineous animals. In addition, since the antibodies developed by Nuria did not display any inhibitory activity with respect to the invasion of Plasmodium falciparum sporozoites, it may be surmised that the observed protection depended on cellular responses, especially on the CTLs.

Example 4: Comparison of the antibody titres before and after immunization with different peptides

4.1. Comparison of the antibody responses induced by different peptides in different immunized animals.

The method used is the one described in Behr et al., (1992), J. Immunol. 149: 3321.

The reactivity is expressed as an ELISA ratio, that is to say the optical density measured at 496 nanometres of the serum after immunization referred to the optical density of the same serum before immunization. The first column shows the animal immunized, the second column the immunogen received by the animal, the 3rd column shows the number of injections carried out as well as the support accompanying the peptide injected: RP denotes recombinant protein, RP/B denotes recombinant protein adsorbed on latex beads, P denotes peptide and LP lipopeptide. It should be pointed out, in addition, that the lipopeptides are injected in physiological saline, the peptides and the recombinant proteins are adsorbed on latex beads or in an emulsion with an adjuvant Montanide ISA-51.

table I: ANTIBODY REACTIVITY OF THE DIFFERENT PEPTIDES EXPRESSED AS AN ELISA RATIO

			LSA-1				SALSA		STARP		LSA-3				
Chimpanzee	Immunogen	Injection No. and type	LSA-REP	LSA-J	LSA-NR	LSA-TER	SALSA -1	SALSA -2	STARP -M	STARP -R	LSA -3- CT1	LSA -3- NR1	LSA -3- NRII	LSA -3- REP	R32T and 32
Immunized animals															
DIRK	LSA-3 and LSA-1	3RP(d)	7.4	9.0	0.9	0.8	nd	nd	0.5	0.7	1.7	1.0	1.1	8.8	0.7
		3RP + 3(P+LP)	20.0	10.0	0.1	0.4	0.2	1.1	1.0	0.6	1.0	1.1	3.1	17.0	0.8
GERDA	LSA-3	3LP	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3.9	nd	0.6
		3LP + 3RP/B	nd	nd	nd	nd	nd	nd	nd	nd	0.7	1.1	3.0	12.3	0.9
DEMI	LSA-3 and LSA-1	2(P+LP)	8.0	14.1	0.7	16.4	0.6	1.1	nd	nd	0.7	1.5	11.7	19.1	0.7
		3(P+LP) 3(P+LP) + 3RP/B	8.4	14.5	1.6	21.5	0.8	0.2	nd	nd	0.8	5.1	14.2	20.7	1.2
KARLIEN	LSA-3 and SALSA	2(P+LP)	0.5	1.2	1.0	1.0	1.1	2.1	nd	nd	1.0	3.6	3.1	10.3	0.9
		3(P+LP) 3(P+LP) + 3RP/B	1.1	0.2	0.5	0.2	1.8	2.5	nd	nd	1.4	4.7	6.8	14.1	0.6
IRIS	LSA-3 and STARP	2(P+LP)	nd	nd	nd	nd	nd	nd	10.1	15.9	0.7	2.4	6.7	12.5	0.6
		3(P+LP) 3(P+LP) + 3RP/B	nd	nd	nd	nd	nd	nd	10.5	16.4	1.3	3.1	6.8	15.3	0.5
Unimmunized controls															
COR	β-GAL	3RP	0.6	0.7	0.8	0.9	0.5	1.0	1.2	0.8	1.1	1.0	0.6	1.1	1.2
PEER	β-GAL	6RP	1.1	0.8	0.7	0.9	0.8	1.2	1.0	0.9	1.1	0.6	0.9	0.9	0.3
BRAM	GST	2RP	1.1	0.6	0.5	1.1	0.3	0.8	0.9	1.2	1.1	0.3	0.4	0.7	1.0
		3RP	0.8	0.3	0.8	1.3	0.7	1.2	1.1	1.2	1.6	0.2	1.3	0.6	0.4
FOUAD	PBS		0.9	0.5	1.0	0.6	0.8	1.3	1.0	0.3	1.9	1.3	0.3	0.2	0.9

4.2. Titre of the antibodies obtained:

Table II shows the antibody titres of the sera obtained in the chimpanzees by immunofluorescence on the native antigens present at the surface of the different stages (sporozoite, liver and blood) of P.falciparum, P.yoelii and P.berghei.

table II: TITRE OF IMMUNOFLUORESCENT ANTIBODIES

		P. falciparum			P. yoelii (17XL and 17XNL)		
CHIMPANZEE	Antigen	SS (NF54)	LS (NF54 and 73OXI)	BS (150)	SS	LS	BS
Immunized animals							
DIRK	LSA-3 and LSA-1	800	200	-(<100)	200	200	-(<100)
GERDA	LSA-3	400	200	-(<100)	400	200	-(<100)
DEMI	LSA-3 and LSA-1	100	400	-(<100)			
KARLIEN	LSA-3 and SALSA	100	200	-(<100)			
IRIS	LSA-3 and STARP	400	100	-(<100)			
Control animals							
COR	β-GAL	-(<100)	-(<100)	-(<100)	-(<100)	-(<100)	-(<100)
BRAM	GST	-(<100)	-(<100)	-(<100)	-(<100)	-(<100)	-(<100)
FOUAD	PBS	-(<100)	-(<100)	-(<100)	-(<100)	-(<100)	-(<100)

4.3. <u>Lymphoproliferative response of the PBMCs of the different chimpanzees</u> after stimulation <u>in vitro</u> either with the different peptides or with the native antigens present at the surface of the sporozoites. This response was measured by incorporation of tritiated thymidine into PBMCs (peripheral blood cells) either after stimulation with the LSA-3 peptides (Table III) or after stimulation <u>in vitro</u> with sporozoites (Table IV).

table III: INCORPORATION OF TRITIATED THYMIDINE INTO PBMCs AFTER STIMULATION WITH THE LSA-3 PEPTIDES

Chimpanzee	Immunogen	LSA-3-CT1	LSA-3-NRI	LSA-3-NRII	LSA-3-REP	MSP3-C (a)	PPD (b)
Immunized animals							
DIRK	LSA-3 and LSA-1	94,256 (4.0)	27,125 (8.5)	32,455 (10.7)	69,321 (32.3)	796 (1.0)	89,338 (50.3)
GERDA	LSA-3	13,359 (25.1)	1,429 (2.8)	13,236 (25.6)	14,883 (28.6)	485 (0.9)	29,355 (132.3)
DEMI	LSA-3 and LSA-1	30,036 (46.8)	17,221 (27.4)	4,178 (7.3)	52,301 (81.2)	689 (1.1)	167,277 (113.3)
KARLIEN	LSA-3 and SALSA	30,025 (36.4)	10,039 (12.8)	18,365 (23.1)	31,312 (38.0)	575 (0.7)	96.212 (82.3)
IRIS	LSA-3 and STARP	53,312 (62.6)	25,223 (34.8)	6,458 (9.7)	35,078 (47.5)	799 (0.9)	196,223 (62.3)
Unimmunized animals							
COR	β-GAL	1,399 (0.6)	2,599 (1.0)	3,625 (1.3)	786 (0.3)	2,600 (1.1)	19,395 (22.3)
PEER	β-GAL	1,225 (0.2)	1,369 (0.3)	3,251 (1.2)	2,960 (0.9)	3,962 (1.5)	59,399 (22.3)
BRAM	GST	1,201 (0.4)	509 (0.2)	2,501 (0.7)	2,659 (0.6)	2,745 (0.7)	39,399 (22.3)
FOUAD	PBS	1,211 (1.2)	1,310 (1.3)	956 (0.9)	688 (0.6)	655 (0.5)	136,258 (82.3)

a) Control peptide from the MSP3 antigen in the blood
b) PPD = Purified protein derivative from Mycobacterium tuberculosis

table IV: INCORPORATION OF TRITIATED THYMIDINE INTO PBMCs AFTER STIMULATION IN VITRO WITH SPOROZOITES

		P.falciparum sporozoites	P. yoelii sporozoites	P. berghei sporozoites
Chimpanzee	Antigen			
Immunized animals				
DIRK	LSA-3 and LSA-1	10,402 (12.1)	5,552 (5.6)	2,110 (2.0)
GERDA	LSA-3	24,021 (20.5)	18,228 (18.6)	2,430 (0.7)
DEMI	LSA-3 and LSA-1	2,111 (3.2)	935 (1.4)	214 (0.1)
KARLIEN	LSA-3 and SALSA	4,402 (6.5)	2,228 (3.6)	914 (2.1)
IRIS	LSA-3 and STARP	9,816 (14.2)	5,304 (8.1)	614 (2.0)
Control animals				
BRAM	GST	245 (0.4)	1,295 (1.6)	514 (1.2)
FOUAD	PBS	997 (1.5)	828 (1.6)	714 (1.1)

The lymphoproliferative responses are shown as a counting difference in counts per minute (Δ CPM) between the number of counts obtained in the presence of antigen minus the number of counts in the absence of antigen. The figures in brackets show the stimulation indices, that is to say the ratio of the number of counts obtained in the presence of antigens to the number of counts obtained in the absence of antigens.

The results are considered to be positive when Δ CPM is greater than 1000 and when the stimulation index is greater than 3.

4.4. Comparison of the antibody responses of chimpanzee Nuria before and after immunization with different peptides

Figure 5 depicts the amounts of immunoglobulins present in the serum of chimpanzee Nuria before and after immunization with the peptides 729NR1 and 729RE, and the lipopeptides 729NR2 and CT1.

This experiment shows the superiority as regards B immunity of the R antigen, most particularly when it is conjugated to a lipid residue.

Figure 6 shows that the level of specific antibodies measured by ELISA against the peptide 729NR2 in mice immunized with either the peptide 729NRII or the lipopeptide 729NRII is markedly higher when the lipopeptide is used, irrespective of the species of mouse.

Example 5: Lymphoproliferation of the PBMCs of an individual protected by injection of irradiated sporozoites against peptides originating from the LSA-1 and LSA-3 antigens

In eight human volunteers immunized by injection of irradiated sporozoites, anti-LSA-3 antibodies are found in each of the four individuals resistant to an infection by sporozoites; and none in the other four volunteers who developed a blood infection.

Furthermore, for the only one of these four protected individuals whose cells were accessible, the PBMCs were removed six months after the challenge

infection and incubated in the presence of the peptides originating from the LSA-1 and LSA-3 antigens.

Figure 10 depicts the results of lymphoproliferation of the PBMCs of an individual protected by injection of irradiated sporozoites against peptides originating from the LSA-1 and LSA-3 antigens.

Considerable lymphoproliferation was observed with each of the three peptides LSA-3 (NR1, NR2 and RE) but with none of the LSA-1 peptides. There was an especially high level of secretion of IFN-γ (100 IU/ml) after stimulation with the peptide NR1 and, to a lesser extent, with the peptide NR2 (IFN-γ: the cytokine having the strongest blocking effect on liver schizogony).

Example 6: Effects of the antibodies against the LSA-3 peptides on the inhibition of the entry of sporozoites in mice

The techniques used to prepare the primary hepatocyte cultures, the sporozoites, the antibodies and the indirect fluorescence test are described in detail by S. Mellouk et al., Bulletin of the World Health Organization, 68: 52-59, 1990. Table V below compares the results obtained in immunofluorescence, either with antibodies against the fragment 679 or with antibodies obtained against fragments originating from other peptides. The left-hand column shows the number of schizonts detected after 48 h of culture in hepatocytes of Balb/c mice infected by P.yoelii and the right-hand column the same parameters after infection by P.berghei.

Table V:

Antibody clones	P.yoelii			P.berghei		
	IFA	No. of LS at 48 h		IFA	No. of LS at 48 h	
		a)	b)			
Control		88	110		119	108
679	++		0	-		47
	++		0	-		ND
679	++	1		-	105	
679b	++	1		-	133	
679c	++	1		-	30	
32	++	8		±	103	
222	+		5	±		26
667	++	276	143	ND	502	
362	+	3				
493	++	55		ND	508	
α P.b. CSP Mab			82	+++		30
α P.y. CSP Mab	+++		171		138	

It is clearly apparent that the antibody against the peptide 679 has an almost complete inhibitory effect on the number of what they [sic] observed at 48 h in the liver cells. Likewise, Figure 7 shows the inhibition of the sporozoite invasion of liver cells by hyperhuman [sic] sera obtained after immunization with different peptides and immunopurified against whole LSA-3.

As regards the protection of mice, the best results were obtained by immunization with the recombinants, or antigens prepared according to the invention, adsorbed on latex or polystyrene microspheres 0.5 µm in diameter:

- 3/3 mice are protected against an administration with 10 times the minimum infectious dose
- 3/3 mice are protected against the second challenge
- 2/3 mice are protected against the third challenge.

The microspheres used are Polybead® polystyrene microspheres (Polysciences, Inc.) 0.50 µm in diameter (ref. 07307) on which the recombinants or the peptides are adsorbed passively. In practice, in mice, per injection, 50 µg of antigens are brought into contact

with 50 µl of microbeads; the exact amount of antigens adsorbed is not determined. In chimpanzees, the same procedure is performed with 200 µg of antigens and 200 µl of beads.

Furthermore, recently, the immunization of mice with the recombinant GST-3PC (corresponding to the non-repeat 3' region from amino acid No. 869 to the stop codon at the 3' end) has enabled sera to be obtained which react very strongly in immunofluorescence with Plasmodium falciparum sporozoites. This result is the first demonstration of the presence of one or several B epitopes in this region of the molecule.

Example 7: Cytotoxicity test against the peptide 729NRII in the chimpanzee Gerda

The chimpanzee Gerda was immunized via the i.v. route with the lipopeptide 729NRII originating from the LSA-3 antigen. Blood is drawn 9 days after the 4th injection. The PBMCs were incubated in vitro with 5 µg/ml of the peptide 729NRII (addition of recombinant IL-2, 10 U/ml, on day 3). On day 15, the cytotoxic activity was studied against autologous blasts generated with PHA at a concentration of 0.5 µg/ml. The blasts were preincubated overnight with 5 µg/ml of the peptide 729NRII, and with a control peptide, namely RESA, or without a peptide. The peptides are not added during the test (8 hours). The number of targets per well is 5000.

PBMCs from Gerda incubated for the same period with 5 µg/ml of a control peptide or the peptide 729NRI (originating from the same antigen) do not bring about the lysis of autologous blasts preincubated or otherwise with the above peptides.

Figure 8 shows the results obtained for an E/T (effector to target) ratio varying from 12 to 0.03. It is seen that the target cells presensitized with the peptide 729NRII are lysed in the presence of effector cells, indicating a cytotoxic T type immune response specific to this antigen.

The lipopeptide NRII injected via the i.v. route is capable, without adjuvant, of inducing a specific cytotoxic response.

Example 8: Effect of the peptide NRI on interferon-γ production

Interferons have been shown to have an inhibitory activity in the development of P.falciparum in human hepatocytes in culture (Sylvie Mellouk et al., The Journal of Immunology, vol. 139 No. 12: 41-92, 41-95, 1987). The results obtained with the peptides of the invention are as follows:

The chimpanzee Gerda, immunized with the polypeptide NR2 and boosted with the recombinant DG729, carries PBMCs capable of secreting high levels of IFN-γ in the presence of the LSA-3 peptides, especially the peptide 729NRI. The result was confirmed in the chimpanzee Dirk, immunized with the same protein. The chimpanzee BRAM, an unimmunized control, does not show any interferon in the blood against the LSA-3 peptides.

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GTGATGAACT TTTTAATGAA TTATTAAA 28

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TGTTGTTCTT GTTGAACACT TTTTACTAA 29

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GGTATCGAAA CTGAGGAAAT AAAGG 25

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CATAGCAGGA ACATCAACAT CCAC 24